IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Alexander Sher et al.
Appl. No.: 09/914,880
Conf. No.: 6145
Filed: April 22, 2002
Title: Iron Fortification System
Art Unit: 1761
Examiner: H. Pratt
Docket No.: 113308-5

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AFFIDAVIT OF ALEXANDER SHER UNDER 37 C.F.R. § 1.132

Sir:

I, Alexander Sher, hereby state(s) as follows:

1. I am one of the named inventors of the above-identified patent application and am therefore familiar with the invention disclosed therein. I have recently reviewed the claims of the patent application as currently pending. A copy of the pending claims is attached hereto as Exhibit A.

2. The present invention generally relates to an iron-protein hydrolysate complex that can be used to fortify foods and beverages with iron. The iron-protein hydrolysate complex as claimed includes ferrous ions chelated to partially hydrolyzed egg white protein with a molecular weight that ranges from about 2,000 to about 6,000.

3. The supplementation of iron sensitive food products with soluble forms of iron is restricted because of the pro-oxidative properties of iron and its ability to produce dark grayish-black chromogens with polyphenols. When attempted, supplementation, has been either with insoluble salts or encapsulated forms. These ingredients, however, have a disadvantage due to poor bioavailability. It is also known that iron complexes which are prepared from intact egg white protein or extensively hydrolyzed egg white protein are not sufficiently strong to prevent discoloration of polyphenol containing beverages/food.

4. The present invention is based on the discovery that partially hydrolyzed egg white protein can strongly complex with ferrous ions and further provide the iron in a bioavailable form. This provides a unique iron source that combines both good functionality (e.g., color, flavor, inhibition of lipid oxidation caused by added iron, strong complex) and high bioavailability.

5. Laboratory trials were conducted that demonstrated the desirable properties of functionality and bioavailability with respect to the iron complex as claimed that includes a specific fraction (e.g., about 2000 to about 6000 molecular weight) of polypeptides derived from a specific protein (e.g., egg white). See, Applicants' Specification, pages 8-14, a copy of which is attached hereto as Exhibit B.

6. It is my understanding that the Patent Office has rejected pending claims 1, 3-8, 11-13, 16-19, 23 and 25-28 as allegedly obvious. Attached hereto as Exhibit C is a copy of the Office Action dated December 28, 2004. Specifically, claims 1, 3-8, 11-13, 16-19, 23 and 25-28 have been rejected under 35 U.S.C. §103 as allegedly unpatentable over British Patent Document No. 673,063 ("Medical Research") and further in view of International Patent Publication No. WO 93/08830 ("Barani") and Kaishi, Vol. 65, No. 11, pages 1635-1641, 1991 ("Kaishi"). A copy of each of these references is attached hereto as Exhibit D.

7. As one skilled in the art, I do not believe that the cited references disclose or suggest the claimed invention. This is true whether the references are viewed alone or in combination. The basis for my opinion is set forth below.

8. Of the cited references, the Patent Office has primarily relied on the Medical Research reference. In my opinion, the Medical Research reference is distinguishable from the claimed invention that recites, in part, an iron-protein hydrolysate complex that includes ferrous ions chelated to partially hydrolyzed egg white protein with a molecular weight that ranges from about 2,000 to about 6,000. At the outset, the focus of the Medical Research reference relates to iron compounds of amino acids. The reference details that "[t]o be of any appreciable metabolic value, iron must be in combination with amino acids . . .". See, Exhibit D, Medical Research, page 1, lines 35-41.

9. As one skilled in the art, the iron compounds of amino acids as provided in Medical Research are different than iron protein complexes, let alone iron protein hydrolysate complexes that include ferrous irons chelated to partially hydrolyzed egg white protein at a molecular weight that ranges from about 2,000 to about 6,000 as claimed. Indeed, the Medical Research publication provides "the preparation of a complex admixture of amino acid salts of iron...by enzymatically digesting a [protein] material to free amino acids therefrom." See, Exhibit D, Medical Research, page 1, lines 54-60. The digestion procedure is carried out to provide a high amino acid yield, thus exhausting the proteins present as far as possible. See, Exhibit D, Medical Research, page 2, lines 18-25.

10. Further, the emphasis of the Medical Research publication relates to the use of casein as the protein substance. The Medical Research publication identifies one specific example for preparing the iron compound with the use of casein. See Medical Research, page 2, lines 57-81.

11. In contrast, the iron complex as claimed includes partially hydrolyzed egg white protein with a molecular weight that ranges from about 2,000 to about 6,000. Again, it was found that iron complexes prepared from intact egg white protein or extensively hydrolyzed egg white protein are not sufficiently stable where iron complexes prepared from partially hydrolyzed egg white protein are extremely stable and further can provide iron in a bioavailable form. In my opinion, the Medical Research publication is distinguishable from the claimed invention based on at least these reasons.

12. The remaining cited references, namely, Barani and Kaishi, do not remedy the deficiencies of the Medical Research reference. The focus of Barani relates to compounds of alleged bioavailable iron with a specific type of an acylated protein where the hydrolysis of such acylated protein is merely optional. See, Exhibit D, Barani, page 5, lines 4-6. Indeed, the sole example in Barani describes an iron compound that is made from an intact acylated protein. See, Exhibit D, Barani, pages 6 and 7. Again, the claimed invention is based on the discovery that partially hydrolyzed egg white protein can strongly complex with ferrous iron and further provide the iron in a bioavailable form.

13. In Kaishi, the primary emphasis relates to the antioxidant activity of hydrolysates of four different proteins that use eight different proteases. Egg white albumin hydrolyzed according to Amino S displayed the highest antioxidant activity. From this hydrolysate, three specific peptides were isolated and their antioxidant effects where compared. See, Exhibit D, Kaishi, Abstract and figures.

14. In my opinion, iron was merely used in Kaishi as a tool to obtain a measure of the purported antioxidant activity. See further, Exhibit D, Kaishi, Abstract and figures. Thus, one skilled in the art in viewing Kaishi would not be led to prepare a stable complex to fortify food stuffs, let alone one that includes iron.

15. Further, the most active peptide (P1) disclosed in Kaishi includes three amino acids. The P1 peptide was purported to be twice as active as both another peptide of similar size (P2) and a much larger peptide (P3). Indeed, the largest P3 peptide has a molecular weight of 927, and thus is well below the lower limit of molecular weight of about 2,000 as claimed.

16. As one skilled in the art viewing Barani and Kaishi, I would not be led to modify the Medical Research reference to provide an iron-protein hydrolysate complex as claimed that includes, for example, a partially hydrolyzed egg white protein at a molecular weight ranging from about 2,000 to about 6,000 based on at least the reasons discussed above. Nowhere would the cited art lead one skilled in the art to predict that an iron complex with any intermediate ligand(s) (e.g., fractions of polypeptides) would form a much stronger complex than with free amino acids or with an intact protein. Further, nowhere would the cited art lead one skilled in the art to predict that a specific egg white protein used for hydrolysis, let alone that a range of polypeptides derived from this specific egg white protein should range from about 2000 to about 6000 molecular weight, to provide an iron complex that is both strong and highly bioavailable. Again, the claimed invention is based on the discovery that an iron complex with a specific polypeptide fraction (e.g., about 2000 to about 6000 molecular weight) derived from a specific egg white protein provides both desirable functionality and bioavailability that Applicants have demonstrated with experimentation as discussed above. Therefore, in my opinion, the cited references, even if combinable, fail to disclose or suggest the iron-protein hydrolysate complex as required by the claimed invention.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made upon information and belief are believed to be true; and further, that these statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any patent that may issue from this Application.

FURTHER AFFIANT SAYETH NOT:

Alexander Sher _____ Date April 27, 2005

Listing of Claims:U.S. Patent Application No. 09/914,880

Claim 1 (previously presented): An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein having a molecular weight in the range of about 2,000 to about 6,000.

Claim 2 (canceled)

Claim 3 (original): A complex according to claim 1 in which the partially hydrolyzed egg white protein is microbial protease hydrolysate.

Claim 4 (original): A complex according to claim 3 in which the microbial protease is obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

Claim 5 (original): A complex according to claim 1 in which partially hydrolyzed egg white protein is a microbial protease hydrolysate obtained by hydrolyzing egg white protein with a protease obtained from *Aseprgillus oryzae* and containing both endo-peptidase and exo-peptidase, and a protease obtained from *Bacillus licheniformis* and containing endo-proteinase.

Claim 6 (previously presented): A complex according to claim 1 which contains about 4.5% to about 10% by dried weight of ferrous irons.

Claim 7 (original): A complex according to claim 1 which is stable at neutral pH but disassociates at a pH below about 3.

Claim 8 (previously presented): An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein which is a microbial protease hydrolyzate; the microbial protease contains both endo-peptidase and exo-peptidase, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000.

Claims 9-10 (canceled)

Claim 11 (previously presented): A complex according to claim 8 which contains about 4.5% to about 10% by dried weight of ferrous ions.

Claim 12 (original): A complex according to claim 8 which is stable at neutral pH but disassociates at a pH below about 3.

Claim 13 (previously presented): An iron-protein hydrolysate complex which comprises ferrous ions chelated to partially hydrolyzed egg white protein; the complex containing about 1% to about 10% by dried weight of ferrous ions, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000.

Claims 14-15 (canceled)

Claim 16 (original): A complex according to claim 13 in which the partially hydrolyzed egg white protein is microbial protease hydrolysate.

Claim 17 (original): A complex according to claim 13 in which the fungal protease contains both endo-peptidase and exo-peptidase.

Claim 18 (original): A complex according to claim 13 which is stable at neutral pH but disassociates at a pH below about 3.

Claim 19 (previously presented): A sterilized liquid beverage which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000.

Claim 20 (canceled)

Claim 21 (previously presented): A sterilized liquid beverage which contains polyphenols and a stable iron foritification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000.

Claim 22 (canceled)

Claim 23 (original): A beverage powder which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein.

Claim 24 (canceled)

Claim 25 (previously presented): A process for preparing an iron fortification system, the process comprising:

enzymatically hydrolyzing an egg white protein using a microbial protease to provide a partially hydrolyzed egg white protein;

adding a ferrous source to the partially hydrolyzed egg white protein under acidic conditions; and

raising the pH to 6.5 to 7.5 for forming a ferrous-hydrolyzed egg white protein complex as the iron fortification system, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000.

Claim 26 (previously presented): A complex according to claim 1 which contains about 1% to about 2% by dried weight of ferrous ions.

Claim 27 (previously presented): A complex according to claim 8 which contains about 1% to about 2% by dried weight of ferrous ions.

Claim 28 (previously presented): A complex according to claim 13 which contains about 4.5% to about 10% by dried weight of ferrous ions.

Claim 29 (previously presented): A sterilized liquid beverage which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000 wherein the sterilized liquid beverage is a chocolate containing beverage.

Claim 30 (previously presented): A sterilized liquid beverage which contains polyphenols and a stable iron foritification system, the iron fortification system comprising an iron-protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein, the partially hydrolyzed egg white protein has a molecular weight in the range of about 2,000 to about 6,000 wherein the sterilized liquid beverage is a tea beverage.

Claim 31 (previously presented): A beverage powder which contains lipid and a stable iron fortification system, the iron fortification system comprising an iron protein hydrolysate complex of ferrous ions chelated to partially hydrolyzed egg white protein wherein the beverage powder contains cocoa.

hence the ability of the complexes to withstand harsh treatment provides a great improvement. However, the complexes may be used in other types of foods or beverages such as powdered beverages, infant formulas, and infant cereals.

The complexes may also be included in pet foods which usually contain lipids and vitamins.

Products which contain the complexes are perceived to have similar organoleptic properties and color as compared to unfortified products. This offers the advantage that products may be fortified without causing noticeable changes which may adversely affect consumer perception. Also, it is found that vitamin C is not degraded by the complexes. Hence the complexes may be used in products which are intended to be nutritionally balanced.

Specific examples of the invention are now described to further illustrate the invention.

Example 1

An amount of 1000 g of frozen egg white is added to a fermentor (Biostat® M) and allowed to thaw at room temperature. The pH is slowly adjusted to 3.0 using 85% H_3PO_4 under agitation. The solution is then heated to 42 °C. An amount of 2.5 g of an acid protease (VALIDASE FP60 obtained from Valley Research, Inc or South Bend, Indiana) is added and the solution allowed to react for 16 hours under low/medium agitation at a pH of 3.0 to 3.3. This acid protease is obtained from *Aspergillus oryzae* and contains both endo-peptidase and exo-peptidase.

After 16 hours of reaction, ammonium hydroxide (28%) is added to raise the pH to 7.4. An amount of 2.5 g of alkaline protease (ALCALASE 2.4L, obtained from Novo Nordisk A7S) is added and the temperature of the solution is raised to 50 °C under agitation. This protease is obtained from a strain of *Bacillus licheniformis* and contains mainly endo-proteinase. After 3 hours of reaction under low/medium agitation, the solution is cooled to room temperature. An amount of 43.5 g of 85% H_3PO_4 is added followed by an amount of 5.0 g of $FeSO_4.7H_2O$ in 50 ml of H_2O, both under agitation. The pH is then adjusted to 6.7 with 28% NH_4OH under agitation. The solution is then heated to a temperature of 90 °C for 10 minutes. The solution is then cooled to room temperature.

The liquid iron complex is collected.

Example 2

The process of example 1 is repeated. Then an amount of 90 g of maltodextrin M.D. 5 is added to the liquid iron complex under agitation. The mixture is then spray dried using an atomizing spinning disk spray-drier (T_{in} = 145 °C, T_{out} = 80 °C).

The powdered iron complex is collected.

Example 3

An amount of 1000 g of frozen egg white is added to a fermentor (Biostat® M) and allowed to thaw at room temperature. The pH is slowly adjusted to 3.0 using 85% H_3PO_4 under agitation. The solution is then heated to 42 °C. An amount of 2.5 g of an acid protease (VALIDASE FP60 obtained from Valley Research, Inc or South Bend, Indiana) is added and the solution allowed to react for 4 hours under low/medium agitation at a pH of 3.0 to 3.3.

After reaction, the solution is cooled to room temperature. An amount of 5.0 g of $FeSO_4.7H_2O$ in 50 ml of H_2O is added under agitation. The pH is then adjusted to 6.7 with 28% NH_4OH under agitation. The solution is then heated to a temperature of 60 °C for 10 minutes. The solution is then cooled to room temperature.

An amount of 90 g of maltodextrin M.D. 5 is added to the solution under agitation. The mixture is then spray dried using an atomizing spinning disk spray-drier (T_{in} = 145 °C, T_{out} = 80 °C).

The powdered iron complex is collected.

Example 4

The process of example 1 is repeated except that the egg white is subjected to hydrolysis for 6 hours. The powdered iron complex is collected.

Example 5

Four chocolate milk beverages are prepared by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) to a concentration of 8.5% by weight.

Each beverage contains 12.5 ppm of added iron in the form of a different iron complex of one of examples 1 to 4.

The beverages are placed into sealed 125 ml glass jars and autoclaved at about 121°C (250°F) for 5 minutes. The jars are cooled to room temperature and stored for 6 months.

The beverages are evaluated for physical stability, color and taste after 1, 2, 3, 4, 5 and 6 months. Taste is judged by a taste panel of 10 people. All beverages are judged to be without discoloration, sedimentation or coagulation and of a good flavor.

Example 6

Four chocolate milk beverages are prepared by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) to a concentration of 8.5% by weight. Each beverage contains 12.5 ppm of added iron in the form of a different iron complex of one of examples 1 to 4.

The beverages are pre-heated to about 80°C (175°F), heated to about 140°C (285°F) by steam injection, held at this temperature for 5 seconds, and cooled to about 80°C (175°F). The beverages are then homogenized at about 17/3.5 MPa (2500/500 psi), cooled to about 16°C (60°F) and filled in 250 ml Tetra Brik Aseptic® packages (Tetra Pak Inc., Chicago IL).

The beverages are evaluated for physical stability, color and taste after 1 day, 2 weeks, and 1 and 2 months. Taste is judged by a taste panel of 10 people. All beverages are judged to be without discoloration, sedimentation or coagulation and of a good flavor.

Example 7

Four chocolate milk beverages are prepared by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) to a concentration of 8.5% by weight. Each beverage contains 12.5 ppm of added iron in the form of a different iron complex of one of examples 1 to 4.

The beverages are pre-heated to about 80°C (175°F), heated to about 148°C (298°F) by steam injection, held at this temperature for 5 seconds, and cooled to about 80°C (175°F). The beverages are then homogenized at about

17/3.5 MPa (2500/500 psi), cooled to about 16°C (60°F) and filled in 250 ml Tetra Brik Aseptic® packages (Tetra Pak Inc., Chicago IL).

The beverages are evaluated for physical stability, color and taste after 1, 2, 3, 4, 5 and 6 months. Taste is judged by a taste panel of 10 people. All beverages are judged to be without discoloration, sedimentation or coagulation and of a good flavor.

Example 8

Six beverages are prepared; 3 by reconstituting a chocolate milk powder (QUIK, Nestlé USA, Inc) and 3 by reconstituting a malted powder (MILO, Nestlé Australia Ltd). Each beverage comprises 22.0 g of powder in 180 ml of boiling water. An iron complex of each of examples 2 to 4 is added to both a chocolate beverage and a malted beverage. The final iron concentrations in the chocolate beverages are 15.0 ppm and in the malted beverages are 25.0 ppm.

The beverage are stirred briefly and allowed to stand for 15 minutes at room temperature. After 15 minutes, beverages are judged by a taste panel of 10 people. No color change or off flavors are found when samples are compared to control samples without added iron.

Example 9

Three infant cereal meals are prepared by reconstituting 55 g of banana containing infant cereal (Nestlé USA, Inc) with 180 ml of boiling water. Iron complexes of examples 2 to 4 added to each cereal to provide 7.5 mg of iron per 100 g of cereal powder.

Each cereal meal is stirred briefly and allowed to stand for 15 minutes at room temperature. After 15 minutes, the cereal meals are judged by a taste panel of 10 people. No color change or off flavors are found when samples are compared to control samples without added iron.

Example 10

The bioavailabilities of the complexes are determined as follows:-

Animals:- The animals used are weanling male Sprague-Dawley rats aged 3 weeks (IFFA-CREDO, L'Arbresle, France).

Diets:- The control diet is an ICN Low-Iron diet (Soccochim SA, Lausanne, Switzerland) which has an iron content of 3 mg/kg. This diet is casein based and provides for the nutritional requirements of growing rats except for iron.

The experimental diets are:-

Diet A:- The control diet supplemented with $FeSO_4.7H_2O$ to provide 10 mg/kg iron.

Diet B:- The control diet supplemented with $FeSO_4.7H_2O$ to provide 20 mg/kg iron.

Diet 1:- The control diet supplemented with the complex of example 4 to provide 10 mg/kg iron.

Diet 2:- The control diet supplemented with the complex of example 4 to provide 20 mg/kg iron.

Diet 3:- The control diet supplemented with 10 mg/kg of the complex of example 2 to provide 10 mg/kg iron.

Diet 4:- The control diet supplemented with 20 mg/kg of the complex of example 2 to provide 20 mg/kg iron.

Analytical methods

1) Hemoglobin analysis is performed by anaesthetizing the rats with isoflurane and then drawing a sample of 200 µL of blood from the orbital venous plexus. Blood hemoglobin level in the sample is determined by the cyanmethemoglobin method (Hb kit MPR 3, Boehringer Mannheim GmbH, Germany), using an automated instrument (Hemocue, Baumann-Medical SA, Wetzikon, Switzerland). Commercial quality control blood samples (Dia-HT Kontrollblut, Dia MED, Cressier, Switzerland) having a range of hemoglobin levels are measured with all hemoglobin determinations.

2) Fe-bioavailability as compared to ferrous sulfate heptahydrate is evaluated using a slope-ratio calculation based upon hemoglobin levels. A multiple regression equation relates amounts of iron added to the hemoglobin

levels. The equation provides one straight line per diet which intercepts at zero dose. The bioavailability of the iron source relative to ferrous sulfate heptahydrate is then calculated as the ratio of the two slopes. The ratio is multiplied by 100 to provide the relative bioavailability value.

Procedure:- Rats are housed individually in polycarbonate cages, fitted with stainless steel grids. The animals are allowed free access to distilled water. To render the rats anemic, the rats have *ad libitum* access to the control diet for 24 days. Fresh diet is supplied daily. Spoiling of diet by rats is reduced by covering the diet with a grid.

After 24 days, hemoglobin and weight is determined. Seventy rats with hemoglobin levels between 4.5 and 5.8 mg/dl are randomized into 7 groups of 10 having approximately equal mean hemoglobin and body weight. Each group of animals is fed one of the experimental diets for 14 days. The rats are fed the diets *ad libitum* beginning with 20 g/day at day 0. The rats have free access to distilled water. Individual food consumption is measured daily. After 14 days, the rats are weighed and hemoglobin is determined.

Results

Mean food consumption and iron intake is not affected by the type of iron source. However the rats receiving no added iron ate less than those receiving iron. The rats consuming diets with 20 mg/kg of added iron consume slightly more than those receiving diets with 10 mg/kg iron.

Weight increase of the rats is not affected by the type of iron source. However, the rats receiving no added iron gained less weight than those receiving iron. The rats receiving diets with 20 mg/kg iron gain slightly more weight than those receiving the diets with 10 mg/kg iron.

The blood hemoglobin levels at the start and at the end of the period are shown in the table below.

Mean hemoglobin values; (Standard Deviation)

Diet	Added Fe (mg/kg)	Initial hemoglobin (g/dl)	Final hemoglobin (g/dl)	Difference (g/dl)
Control	0	5.12 (0.42)	4.88 (0.43)	-0.24 (0.20)
A	10	5.12 (0.41)	8.66 (0.81)	3.54 (0.65)
B	20	5.12 (0.40)	11.53 (0.86)	6.41 (0.82)
1	10	5.12 (0.40)	7.90 (0.54)	2.78 (0.41)
2	20	5.13 (0.39)	11.15 (0.57)	5.92 (0.54)
3	10	5.13 (0.37)	8.36 (0.47)	3.23 (0.34)
4	20	5.12 (0.38)	11.51 (0.79)	6.39 (0.65)

The relative bioavailabilities are as follows:-

Diet	Relative Bioavailability
1, 2	90
3, 4	98
A, B	100

The bioavailabilities of all of the Fe-protein complexes are similar to that of ferrous sulfate. A relative bioavailability value of less than 91% is taken to be significantly less than the reference. Therefore, from a statistical point of view, the relative bioavailability values of the iron complexes of example 2 are similar to that of ferrous sulfate. However, from a practical viewpoint, all of the complexes have very good bioavailability.

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EXAMINER	
PRATT, HELEN F	

ART UNIT	PAPER NUMBER
1761	

DATE MAILED: 12/28/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	09/914,880	SHER ET AL.	
	Examiner	Art Unit	
	Helen F. Pratt	1761	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *09 September 2004*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,3-8,11-13,16-19,21,23 and 25-31* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☒ Claim(s) *29-31* is/are allowed.

6)☒ Claim(s) *1,3-8,11-13,16-19,23 and 25-28* is/are rejected.

7)☐ Claim(s) *21* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 21 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claim 21 is redundant with claim 30.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1, 3-9, 11-13, 16-19, 23, 25-28 are rejected under 35 U.S.C. 103(a) as being unpatentable over Medical research (673,063) in view of Barani et al. and Kaishi.

Medical research discloses that it is known to make an iron-protein hydrolysate out of egg albumin (egg white) (page 2, lines 85-95). Claim 1 differs from the reference in the particular molecular weight (mw). However, nothing is seen and nothing has been shown that molecular weights within the claimed range are not used in the composition of the combined references. The independent claims contains the limitation that the molecular weight is in the range of about 2,000 to 6,000. However, nothing is seen that the reference to MR does not contain these weights because the protein is hydrolyzed to polypeptones and the like and unconverted proteins (page 2,

lines 10-16). It would have been expected that since the claimed process has been disclosed that the claimed molecular weights would have been present. In addition, Barani et al. disclose a composition which contains an iron-protein hydrolysate complex made from ferrous ions and hydrolyzed egg white protein (abstract and page 3, lines 5-13, lines 25-30, and page 4, lines 25-32 and page 5, lines 4-8). Kasihi (XP-000914255) discloses that it is known to make an iron and egg-white albumin hydrolyzate (abstract, last 5 lines). In addition, nothing critical is seen in the higher molecular weights, as 500 had previously been claimed. Therefore, it would have been obvious to make an iron-protein hydrolysate complex with various molecular weights.

Claim 3 requires that the egg protein is hydrolyzed with a microbial protease. Kaishi discloses that the hydrolysis was done using Bacillus subtilis, which is a microbial protease, which would produce a microbial protease hydrolysate (abstract). Therefore, it would have been obvious to make a microbial hydrolysate as claimed.

Claims 4-5 require particular proteases. However, as one protease has been disclosed which makes a hydrolyzate, it would have been within the skill of the ordinary worker to use other protease, absent anything new or unobvious being produced. Therefore, it would have been obvious to use other proteases to make the claimed composition.

Claim 6 requires that the complex contain particular amounts of ferrous ions and claim 7 that it is stable at a neutral pH, but disassociates at a pH below 4. It is seen at this time that the above composition from the various references do contain the claimed about of ferrous ions. Barani et al. disclose that the iron content is 11%, which is a little

more than the claimed amount (page 6, lines 24-25). No patentable distinction is seen in the use of 10% and 11 % at this time. Therefore, it would have been obvious to use particular amounts of iron in the composition as shown by Barani et al. Medical research discloses a pH of amount 3-6 (page 2, lines 25-30). It is not known whether the composition disassociates at a pH of below 3. Since the claimed composition has been shown at the right pH, it is seen that it would have disassociated at below a pH of 3. . Therefore, it would have been obvious to use the teachings of the references to Barani et al. and Kaishi in the composition and process MR because these references are to various aspects of hydrolyzed proteins.

Therefore, it would have been obvious to make a product as claimed as shown by the above references.

The limitations of claims 8, 11-13, 16-18 have been disclosed above and are obvious for those reasons.

Claim 19 further requires that the iron protein hydrolysate complex is a sterilized liquid. Nothing new is seen in sterilizing liquids, which is within the skill of the ordinary worker. Therefore, it would have been obvious to sterilize the claimed composition.

Claim 23 is to a beverage powder containing lipids. However, fortified beverage powders are well known as is fat in food composition. The reference to Barani discloses that it is known to use iron in foods (page 2, 17-21). Powders are disclosed in page 5, lines 25-29). Therefore, it would have been obvious to use the claimed composition in a powder.

The limitations as to the process have been disclosed by Medical Research as in claims 25-28, who uses an enzymatic agent to digest proteins which can be egg white (albumin (page 2, lines 25-56, page 1, lines 80-85, page 2, lines 25-30, lines 50-55). Therefore, it would have been obvious to make a composition as claimed.

Allowable Subject Matter

Claim 21 would be allowable if rewritten or amended to overcome the rejection(s) under 35 U.S.C. 112, 2nd paragraph, set forth in this Office action.

Claims 29-31 are allowed.

ARGUMENTS

Applicant's arguments filed 4-26-04 have been fully considered but they are not persuasive. Applicants argue that Applicants invention is defined by a particular molecular weight. Nothing has been shown that the hydrolyzed protein of MR does not disclose the claimed molecular weight, as in polypeptones, peptides and unconverted proteins (page 2, lines 12-20 and lines 30-34).

Applicants argue that MR is to preparing complexes of free amino acid and iron. However, the reference is not limited to free amino acids as discussed above. The specification on page 6, lines 3-5 does not show any comparison between the degree of hydrolyzation of egg white to define over the references.

It is not seen that a laundry list of materials can be used in MR, but various proteins in a group of 6. The composition of the reference is said to have been more effective than previous iron compositions and uses less of the composition to administer iron.

Applicants argue that Barani is to acylation and hydrolysis is optional. However, the reference discloses acylated hydrolysis or the other (page 5, lines 4-6). The method steps of Barani are not considered in a composition claim, and have not been excluded from the claimed method.

Applicants argue as to Kaishi that that it is to showing the antioxidant levels of hydrolysates. Even so, this does not exclude the fact that it is known how to make an iron-egg-white albumin hydrolyzate and is not limited to the most active peptide.

It is seen that the claimed product and process has been disclosed by the combined references, absent a showing that the claimed weight of the hydrolyzed proteins of MR are not within the claimed range.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Helen F. Pratt whose telephone number is 571-272-1404. The examiner can normally be reached on Monday to Friday from 9:30 to 6:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mr. Milton Cano, can be reached on 571-272-1398. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should

you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Hp 12-23-04

H. Pratt
HELEN PRATT
PRIMARY EXAMINER

PATENT SPECIFICATION

673,063

Date of Application and filing Complete Specification: June 16, 1948.
No. 16257/48.
Application made in Australia on June 23, 1947.
Complete Specification Published: June 4, 1952.

Index at acceptance :—Class 2(iii), C2b2, H.

COMPLETE SPECIFICATION

Metallic Compounds of Amino Acids and Preparation thereof

We, MEDICAL RESEARCH PROPRIETARY LIMITED, a company incorporated in the State of New South Wales, Commonwealth of Australia, of Scot Chambers, Hosking Place, Sydney, New South Wales, Australia, do hereby declare the nature of this invention and in what manner the same is to be performed, to be particularly described and ascertained in and by the following statement:—

In the past many different organic compounds of iron have been incorporated in foods and have otherwise been prepared for medical purposes. Examples of such compounds are those of iron and ammonium citrate, thio-compounds in conjunction with organic acid radicals, ferrous and ferric gluconates and the like. As such compounds are not in a highly biochemically active form, it has been necessary to administer them in very large doses, in some cases up to 90 grains per day. By this invention organic compounds of iron, are prepared in a highly active form, that is, in a form which very closely approximates to the form in which iron is contained in blood hæmoglobin or other body fluids. It follows that advantages due to the present invention are that dosages may be considerably reduced, by comparison with what has been regarded as normal dosage heretofore, due to the compounds subject hereof being much more readily and much more completely capable of assimilation.

To be of any appreciable metabolic value iron must be in combination with amino acids, and it is believed that in so far as iron has been capable of assimilation by humans or animals it is because it is converted to this amino acid form within the alimentary system. So far as is known, iron compounds of all the amino acids derivable from a proteinous material have not hitherto been synthesised as a readily assimilable group other than within a living human or animal body as a metabolic function thereof. The present invention has been devised to synthesize iron compounds with all the amino acids obtainable from a common proteinous material thus to provide an admixture of amino acid salts which closely approaches the complex amino acid salt admixture present in natural hæmoglobin.

The method subject hereof consists in the preparation of a complex admixture of amino acid salts of iron in a form substantially the same as that of the amino acid salts of iron occurring in natural hæmoglobin, comprising the steps of enzymatically digesting a proteinous material to free amino acids therefrom; and, without separating any of said acids, reacting the entire admixture thereof with an iron compound selected from the group consisting of the hydroxides, hydrated oxides and carbonates of iron. The reaction of the amino acid admixture with the iron compound may be carried out at ordinary room temperatures, but for preference (in order to shorten the reaction time) the reaction is carried out at an elevated temperature, of the order, for example, of 60–80° C. If desired, the step of reacting the amino acids with the iron compound may consist in first reacting the amino acid admixture with a soluble salt of sodium or other metal other than iron, and then forming the required complex admixture of amino acid salts by double decomposition between the amino acid salts of the metal other than iron with an iron salt.

Practically any protein substance may be employed as starting material herein. Such substance may be casein, gelatine, yeast, soya bean flour, egg albumen, vegetable or animal albumen or a mixture of two or more such protein substances in any proportions.

The ph value of the protein is first adjusted in accordance with the nature of the protein used and the enzymes to be used in the digestion.

The digestion may be carried out under acid, neutral or alkaline conditions. With acid digestion pepsin is added to the proteins as an enzymatic or catalytic agent. The

amount of pepsin may vary from the merest trace up to as much as or more than 10 per cent by weight of the proteins. In the case of neutral digestion the preferred enzymatic or catalytic agent is papain used in the same quantities as indicated above, with alkaline digestion the preferred enzymatic agent is pancreatic extract also in the same proportions. After addition of the enzymatic agent the temperature of the batch is adjusted to give optimum enzymatic action. This temperature may be from about 35° to 40° C. Digestion is then allowed to proceed, giving an admixture of amino acids, polypeptones and the like, and unconverted proteins. It will be understood that depending on the progress of the digestion and the nature of the proteinous matters used, the digestion may be carried out in several stages, in some of which the ph value may be varied so that a digestion which commenced as an acid process is carried on as a neutral or alkaline process, the procedure being varied to give a high amino acid yield; and, as far as possible, to exhaust the proteins present. The mixture resulting from digestion then has its ph value adjusted to from 3·6 to 4·6 (preferably about 4), that is, if it is not already of about this hydrogen ion concentration. This frees the amino acids from combination. For many medicinal uses it is not harmful for the salts, peptides, unconverted proteins and other matters to remain with the amino acids.

To produce an iron compound or compounds with the amino acids, a soluble salt or salts of iron is or are converted to hydrate, hydroxide or carbonate form. The hydroxide form is preferable and may be brought about by addition of caustic soda or other alkali. In either case this action gives a precipitation which, if required, enables unwanted salts or the like to be removed. Alternatively, such salts may be allowed to remain if they are biochemically unobjectionable or in some cases medicinally desirable (as may be the case where glauber salts or other medicinal agents are formed). The iron hydrate, hydroxide or carbonate is then mixed with the amino acids still in accompaniment with unconverted proteins, peptones and the like in such proportions as will give the resultant mixture a ph value equal or about equal to from 6·5 to 7. This admixture is sufficient to give the required iron compounds of the amino acids. A typical example of the invention is set forth below.

EXAMPLE

MANUFACTURE OF A COMPLEX BIOLOGICALLY ACTIVE IRON COMPOUND

5 pounds of casein, 2 pounds of gelatine, and 70 pounds of water are mixed and the resulting solution subjected to enzymatic hydrolysis by the addition of approximately 20 grammes of pancreatic extract.

Sufficient sodium carbonate is added to adjust the ph to between 7·3 and 8·0 and the whole maintained at a temperature of between 38/40° centigrade until hydrolysis has reached a stage where between 60 and 70% at least of the protein is hydrolized.

The above solution is then adjusted to a ph 4·3 by the addition of sulphuric acid, when a previously prepared hydroxide of iron is added to the amino acid hydrolysate and the whole thoroughly agitated and maintained at a temperature of between 60 and 80° centigrade (preferably under vacuum) until ph of 6·5 to 7·0 is obtained.

The clear solution obtained after filtration is concentrated to any degree or to dry powder form as required.

Having now particularly described and ascertained the nature of our said invention and in what manner the same is to be performed, we declare that what we claim is:—

1. A method of preparing a complex admixture of amino salts or iron in a form substantially the same as that of the amino acid salts of iron occurring in natural hæmoglobin, comprising the steps of enzymatically digesting a proteinous material to free amino acids therefrom, and, without separating any of said acids, reacting the entire admixture thereof with an iron compound selected from the group consisting of the hydroxides, hydrated oxides and carbonates of iron.

2. A method according to Claim 1, wherein the said step of reacting an entire admixture of amino acids with an iron compound is carried out at an elevated temperature.

3. A method according to Claim 1, wherein the said step of reacting an entire admixture of amino acids with an iron compound, consists in first reacting the amino acids of said admixture with a soluble salt of a metal other than iron, and then forming the required complex admixture of amino acid salts by double decomposition between said amino acid salts of a metal other than iron with an iron salt.

4. A method according to Claim 1, wherein the step of digesting a proteinous material is performed under alkaline conditions and by use of pancreatic extract as enzymatic agent.

5. A complex admixture of amino acid salts of iron when prepared by a method according to any of the preceding claims.

Dated this 16th day of June, 1948.

MEDICAL RESEARCH PROPRIETARY LIMITED,
By:
ERIC POTTER & CLARKSON,
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317, High Holborn, London, W.C.1.

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(54) Title: COMPOUNDS OF BIOAVAILABLE IRON WITH ACYLATED OVOTRANSFERRIN OR WITH ACYLATED HYDROLYSIS DERIVATIVES THEREOF

(57) Abstract

Compounds of bioavailable iron with acylated ovotransferrin or with acylated hydrolysis derivatives thereof, useful in the treatment of iron deficiencies.

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COMPOUNDS OF BIOAVAILABLE IRON WITH ACYLATED OVOTRANSFERRIN OR WITH ACYLATED HYDROLYSIS DERIVATIVES THEREOF

The present invention relates to compounds of bioavailable iron with acylated ovotransferrin or with acylated hydrolysis derivatives thereof.

Iron, which is present in all the body tissues, plays a paramount physiological role. The iron requirement is satisfied partially by the use of endogenous iron, deriving from the degradation of old erythrocytes, and partially from the absorption of exogenous iron.

Exogenous iron is absorbed along all the duodenum and the upper part of jejunum and it is accumulated mainly in the liver.

The first pathological symptom of iron deficiency is hypochromic sideropenic anemia, whose primary causes can be of various origin: chronic hemorrhages occurring in case of gastroduodenal ulcers or neoplasias; an insufficient diet or a bad absorption, as in the case with diarrhoea; increased requirements, for example during pregnancy, lactation, infectious diseases and the like; impaired metabolic utilization; particular treatments, such as with ACTH or cortisones.

The administration of iron proved to effectively reduce the iron-related anaemic condition, but it is generally accompanied by undesired side-effects, which are related to the type of vector used for the iron.

The ferro-dextran complex has been suggested for the intramuscular administration, whereas the ferro-

dextrin complex is used for the intravenous administration. The side effects of both said complexes can be allergic reactions, temperature rises, tachycardia, leukocytosis, lymphoadenopathy, in the case of intramuscular treatment, and even anaphylactic shock, thrombophlebitis and circulatory collapse in the case of intravenous treatment.

In the per os treatment, formulations are used based on organic salts (citrate, cholinate, aspartate, gluconate, glycinate, lactate, oxalate, succinate etc.) or inorganic salts (ferric chloride, ferrous sulfate, ferric phosphate etc.) which generally lead to gastrointestinal lesions with necrosis and perforation of the mucous membranes in the most serious cases, and diarrhoea and vomiting. Moreover, the low tolerability makes the administration of suitable amounts of iron difficult. In order to minimize the side effects, the simultaneous intake of food has been suggested, but this is in contradiction with the proven variability of the iron absorption as a function of the composition of food itself and of the degree of the gastric contents.

An alternative to the use of said salts in the oral therapy has been provided by the commercialisation of specialties based on ferritin, which is a ferric globulin representing the most important iron-containing protein in mammals. The commercial product is extracted from horse spleen as a raw material. Ferritin has a 20% iron content in terms of dry weight, it is water soluble and suitable for the oral administration. Ferritin based treatment does not involve the gastrointestinal side effects arising

during the use of the above mentioned iron derivatives, but it has severe restrictions deriving both from the very high cost of the raw material and mainly from the limited availability of extraction sources.

Therefore an attempt was carried out to use other proteins from animals (serum proteins, organ proteins, ovoalbumin, lactoproteins) or from vegetables (soy proteins) as iron carriers. However, the interaction between ferric salts and the above mentioned proteins leads to the formation of ferro-protein derivatives whose therapeutic interest is undermined by a series of negative characteristics, including:

- the insolubility of the derivatives obtained when the percentage of iron linked to the protein reaches values greater than 0.5%;
- the difficulty or even the impossibility of evaluating what fraction of the total iron content, under such conditions of insolubility, is actually linked to the protein and what fraction is co-precipitated in the form of hydrated oxides which may cause severe gastric lesions;
- the lack of homogeneity and compositional stability of these derivatives with respect to iron.

Subsequently, it has been found (see Italian Patent n. 1150213 in the Applicant's name) that, by carrying out a succinylation of the above mentioned proteins and reacting them with iron, ferroprotein derivatives could be obtained which have a fairly good iron content, are stable and sufficiently soluble at pH values above 5, and are able to supply therapeutically

acceptable iron concentrations when administered orally. However, since said proteins have a varying composition, it is very difficult to obtain compounds having a constant iron content. Moreover, even though compounds can theoretically be obtained with a fairly good iron content (up to 20%), such an iron content involves an increase in the viscosity of the solution of said products, therefore up to now such products are commercialized having an iron content of only 5% by weight.

WO 91/07426 discloses a very soluble iron-acylated albumin compound, but, even though the use of different types of albumin is stated to be effective, the best results are achieved with bovine serum-albumin, which yields a compound with a 10% iron content, above which value the solubility of the compound decreases, thus lowering the therapeutic value. Recently, the use of all of the products of bovine origin has severely been restricted by the dramatic problem of the virus of bovine spongiform enkephalitis (BSE) which has already caused some therapeutically interesting substances, such as ferritin, to be withdrawn from the market.

As a consequence, the interest of researchers has been focalized on proteins of a different origin.

Now it has surprisingly been found that, among all of the proteins useful as iron carriers in the martial therapy, ovotransferrin (also named conalbumin), suitably acylated, gives compounds having a higher iron content than other ferro-protein compounds, while keeping those viscosity and solubility parameters which make therapeutically acceptable the compound, therefore

ensuring a larger iron supply to the patient without any of the undesired side effects typical of said therapy occurring.

The present invention relates to compounds of bioavailable iron with acylated ovotransferrin or with acylated hydrolysis derivatives thereof.

Preferably, the acyl moiety of the compound consists of a dicarboxylic acid derivative such as malonic, succinic, methylmalonic, ethylmalonic, acetylmalic, acetylglutamic, acetylaspartic, glutaric acids and the like. Preferred carboxylic acid derivatives are the succinic and acetylaspartic acid derivatives.

The compounds of the present invention have an iron content from 3 to 20% by weight. Preferably the iron content of said compounds is 11% by weight at least.

Said compounds are suitable as active ingredients for the preparation of pharmaceutical compositions which can be used in the oral treatment of anemias and in all the pathological conditions caused by an iron lack in mammals and in man. Therefore another object of the invention is provided by the use of the present compounds for the preparation of medicaments useful in said pathologies. Pharmaceutical forms suitable for the oral administration of the compounds of the present invention are, for example, tablets, sugar-coated tablets, capsules, powders, granulates, syrups, suspensions and solutions.

The present invention is illustrated in further detail by the following non-limiting example.

EXAMPLE

5 g of ovotransferrin are dissolved in 100 ml of water containing 3 g of $KHCO_3$, the clear solution is added with 2.5 g of succinic anhydride, in subsequent portions and adjusting pH to values ranging from 5 to 8 by addition of NaOH. The mixture is left to react for 2 hours at room temperature, then, after acidification to pH 3.4, a precipitate is obtained which is recovered by centrifugation, purified adjusting pH to 7.5 by addition of NaOH and subsequently reprecipitated at pH 3.4. By centrifugation a solid is recovered which is dried under vacuum. The dry solid is resuspended in distilled water and dissolved by addition of NaOH to pH 8, to obtain a final solution of 0.04 g of protein/ml.

Said solution, having a very high viscosity, is added with a solution of ferric chloride so as to obtain a weight ratio of succinylated protein to Fe^{3+} of 10:1. Under said conditions, pH decreases to 2.6 and a precipitate forms which is recovered by filtration, then redissolved in water and added with NaOH until complete dissolution (pH 7.5). After dialysis against water to remove sodium chloride, the solid product is recovered by lyophilization.

The compound yield is 33% by weight of the starting protein and the iron content is 11%.

The product of the above example was administered orally to groups of rats with strong sideropenic anemia, experimentally induced by feeding the animals with an iron-free diet from the pre-natal time to the one of the test. The administered compound dose was 1 mg/kg iron.

A group was treated with placebo. One or two hours after the treatment, the animals were killed with ether, the blood was collected, the serum was prepared and sideremia was evaluated by means of a commercial kit. The table below summarizes the means ± S.E. of the values obtained in 6 animals.

Treatment	Withdrawal time	Serum Fe ug/100 ml
Placebo	1 hour	60.9± 3.3
Fe ovotransferrin	1 hour	358.2±30.9
Fe ovotransferrin	2 hours	484.9±46.4

CLAIMS

1. A compound of bioavailable iron with acylated ovotransferrin or with an acylated hydrolysis derivative thereof.

2. A compound according to claim 1, wherein the acyl moiety is a dicarboxylic acid derivative.

3. A compound according to claim 2, wherein the dicarboxylic acid derivative is a succinic or an acetylaspartic acid derivative.

4. A compound according to claim 1, wherein the iron content is from 3 to 20% by weight.

5. A compound according to claim 1 or 4, wherein the iron content is at least 11% by weight.

6. The use of the compound of claim 1 for the preparation of medicaments useful in the iron deficiencies.

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/14

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOCHEMISTRY vol. 4, no. 6, June 1965, EASTON, PA US pages 998 - 1005 H. BUTTKUS, 'Chemical modifications of amino groups of transferrins: ovotransferrin, human serum transferrin and human lactotransferrin.'	1-5
Y	see the whole document	6

X	BIOCHIM. BIOPHYS. ACTA vol. 181, 1969, pages 295 - 304 A. BEZKOROVAINY, 'Some physical-chemical properties of succinylated transferrin, conalbumin and orosomucoid.'	1-5
Y	see the whole document	6

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[10] Special categories of cited documents:

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 FEBRUARY 1993	10. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ORVIZ DIAZ P.

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	POULTRY SCIENCE vol. 61, no. 6, 1982, pages 1041 - 1046 H.R. BALL, 'Acylation of egg white proteins with acetic anhydride and succinic anhydride.'	1-5
Y	see the whole document	6
Y	US,A,4 493 829 (G. SPORTOLETTI) 15 January 1985 see the whole document, especially see claim 12; example 4 & IT,A,1 150 213 (cited in the description)	1-6
Y	WO,A,9 107 426 (ITALFARMACO S.P.A) 30 May 1991 see claims (cited in the description)	1-6
Y	EP,A,0 319 664 (ITALFARMACO S.P.A.) 14 June 1989 see claims; examples	1-6
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ON INTERNATIONAL PATENT APPLICATION NO. EP 9202489
SA 66712

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4493829	15-01-85	AT-B-	390067	12-03-90
		AU-A-	1185883	08-09-83
		BE-A-	896051	01-07-83
		CA-A-	1222508	02-06-87
		CH-A-	653893	31-01-86
		DE-A,C	3306622	15-09-83
		FR-A,B	2522664	09-09-83
		GB-A,B	2115821	14-09-83
		JP-B-	4070317	10-11-92
		JP-A-	58159421	21-09-83
		LU-A-	84672	08-09-83
		NL-A-	8300757	03-10-83
		SE-B-	462716	20-08-90
WO-A-9107426	30-05-91	AU-A-	7039691	13-06-91
EP-A-0319664	14-06-89	JP-A-	1146900	08-06-89

報　文

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卵アルブミンの酵素分解によって得られるペプチドの抗酸化性について

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Antioxidative Activity of Peptides Prepared by Enzymatic Hydrolysis of Egg-white Albumin

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The antioxidative activity of protease hydrolyzates of four proteins against linoleic acid was investigated in an aqueous system at pH 7.0. Eight kinds of protease were used. The egg-white albumin hydrolyzate prepared with Amano S (from *Bacillus subtilis*) had the strongest antioxidative activity. From this hydrolyzate, three antioxidative peptides were purified by Sephadex G-25 gel filtration, CM Sephadex C-25 column chromatography, and then HPLC on an octadeceyl column. The amino acid sequences of the peptides were Ala-His-Lys (P1), Val-His-His (P2), and Val-His-His-Ala-Asn-Glu-Asn (P3). The antioxidative activity of P1 was the strongest, and the activities of P2 and P3 were about half that of P1. A mixture of Fe^{2+} and egg-white albumin hydrolyzate was separated by Sephadex G-25 gel filtration, and the recovery of Fe^{2+} was measured. Most of the bound iron was eluted in the fractions corresponding to the peak of the antioxidative activity. This suggests that the antioxidative activity was based on the chelating activity of the peptides.

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緒　言

近年，抗酸化成分が種々の天然物から単離され，その構造解析より，化学構造と抗酸化作用との関係が明らかにされてきている[1,2]．また，これらの抗酸化成分については，老化や発ガンなどの関連から，食品の第三次機能の面も期待され，活発に研究が行われている[3]．

水溶性の抗酸化剤としては，アミノ酸や蛋白質について多くの研究が行われている．アミノ酸や蛋白質が発揮する抗酸化能が，金属キレート作用に起因しているということは，梶本ら[4~7]をはじめ多くの研究者によって報告されている[8~10]．また，アミノ酸やペプチドとラジカルスキャベンジャー的な作用を示す抗酸化成分と併用されている[11]．したがって，このことからもアミノ酸や蛋白質の抗酸化効果の主体が金属キレート作用であることを支持している．

一方，山口ら[11~14]は，一部のジペプチドがアミノ酸や蛋白質よりも抗酸化力が強いことを報告しており，特定の組合わせのジペプチドが遊離のアミノ酸よりも抗酸化力が増大することは興味深い．しかし，ジペプチドから蛋白質に至るポリペプチドのアミノ酸配列と抗酸化力に関する知見はきわめて少ない．

そこで著者らは，抗酸化力とアミノ酸配列の特長を明らかにし，さらにそれらのペプチドの抗酸化作用を推定するために，種々の蛋白質の酵素分解物から抗酸化力の強いペプチドを単離し，その構造を調べた．

実 験 方 法

1. 実験材料　（1） 蛋白質と酵素：大豆蛋白質：和光純薬工業製，小麦グルテン：東京化成工業製，卵製アルブミン：半井化学製，牛血アルブミン：SIGMA 社製，ビオプラーゼ：ナガセ生化学工業製，プロチン：大和化成製，プロンザー：天野製薬製，ニューラーゼ：天野製薬製，アマノS：天野製薬製，アマノN：天野製薬製，ペプシン：和光純薬工業製，α-キモトリプシン：生化学工業製．

（2） 試薬：リノール酸は，半井化学製，純度 99% 以上の特級品を使用した．その他の試薬は，すべて市販の特級試薬を用いた．

2. 測定方法　（1） 蛋白質の酵素分解の条件[13]：おのおの蛋白質 5 g を脱イオン水に懸濁し，沸騰水浴中で 20 分間加熱後放冷して，各種酵素製剤 200 mg を添加した．溶液を，おのおの酵素の至適 pH に調整し，35°C で 48 時間反応させた．48 時間後に，溶液を濾過し酵素を加熱失活させ，pH を中性に戻してふたたび濾過した．

（2） ゲル濾過クロマトグラフィー：Sephadex G-25 を脱イオン水で膨潤させた後，カラム（φ 25×500 mm）に充填し，試料 250 mg をチャージして脱イオン水で溶出した．流速は 2 ml/10 min で，各フラクションは 5 ml ずつ分取し 280 nm の吸光度を測定した．

（3） イオン交換クロマトグラフィー：CM-Sephadex C-25 を（2）と同様にしてカラム（φ 20×300 mm）に充填した．試料は脱イオン水と 0.5 M 塩化ナトリウム水溶液の濃度勾配を用い，流速 5 ml/10 min で溶出させた．各フラクションを 5 ml ずつ分画し 220 nm の吸光度を測定した．

（4） 高速液体クロマトグラフィー（HPLC）：HPLC は東ソー製，8010 シリーズを使用し，ODS カラム（TSKgel ODS-120 T 4.6 mm×25 cm）を用いた逆相系で，0.1% トリフルオロ酢酸（TFA）を含む脱イオン水とアセトニトリルの濃度勾配で溶出し，220 nm の吸光度を測定してペプチドの溶出位置を求めた．

（5） 単離されたペプチドのアミノ酸配列の測定：アプライドバイオシステムズジャパン製 Model 477 A シーケンサーを用いて測定した．

（6） 抗酸化力の測定[13,15]：エタノール 10 ml，0.1 M リン酸緩衝液（pH 7.0）10 ml，脱イオン水 5 ml の混合液にリノール酸を最終濃度として 2×10^{-2}M になるように添加した．この溶液を 50 ml 容バイアル瓶に入れ，密栓して 60°C 恒温器に放置した．なお，試料の添加量に応じて上記脱イオン水を減じ，全量は常に 25 ml とした．

このバイアル瓶から経時的に 0.1 ml を採取して，ロダン鉄法[10]によって過酸化物量を測定した．その際，500 nm の吸光値が 0.3 に達するまでの日数を誘導期間とした．

（7） 金属キレート能の確認[16]：Sephadex G-25 カラム（φ 25×500 mm）を，0.1 mM $FeSO_4$ ($CuSO_4$, $ZnSO_4$) を含む 0.07M 酢酸緩衝液（pH 4.0）で平衡化させた後，卵アルブミンのアマノS分解物を負荷して同じ緩衝液で溶出した．流速は 5 ml/min で，各フラクション 5 ml ずつ分取した．展開後，各フラクションの金属含量を，島津製作所製 ICPS-50 で測定し，同時に 280 nm の吸光度を測定した．

（8） ラジカル捕捉能の確認：フェノール試薬は，脱イオン水で2倍に希釈して用いた．1,1-ジフェニル-2-ピクリルヒドラジル（DPPH）は，少量のアルコールで溶解した後，0.01% 濃度の試料液 1 ml に 2,3 滴加えて反応を確認した．なお，ラジカル捕捉能が存在するときは，フェノール試薬は黄色から青紫色に，DPPH は紫色から無色に反応する．

実験結果および考察

1. 抗酸化力を持つ蛋白質の酵素分解物のスクリーニングについて

蛋白質 4 種類と酵素 8 種類で調製された蛋白質の酵素分解物 32 種類について抗酸化力を調べた．抗酸化性の認められたものは，酵素としてアマノS，アマノNを用いたもので Fig. 1 に示すような結果となった．

蛋白質として卵アルブミン，大豆蛋白質，小麦グルテン，酵素としてアマノSを用いたものは，対照区やその他の分解物に比較して抗酸化力が著しく強く，約 50 日以上の誘導期間が認められた．このことからペプチドの抗酸化作用には，蛋白質の種類に基づくペプチドの構成アミノ酸や，酵素の基質特異性による末端アミノ酸が影響していることが考えられる．

2. 抗酸化活性の強いペプチドの単離について

次は，スクリーニング結果から，最も抗酸化活性の強い卵アルブミ

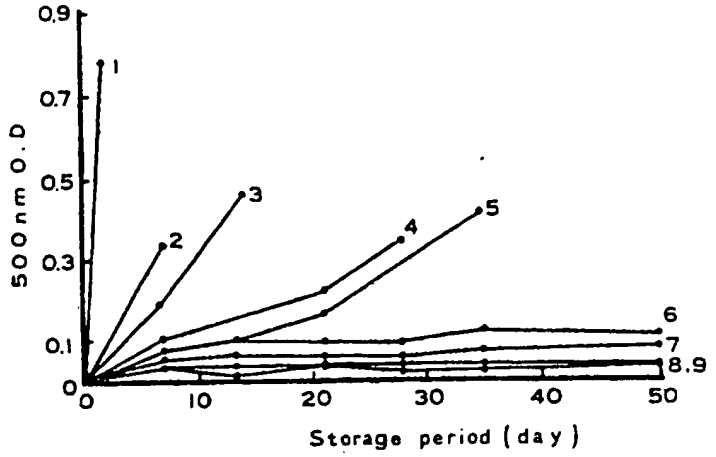

Fig. 1. Effect of Various Protein Hydrolyzates on Autoxidation of Linoleic Acid in Aqueous System. Test vial was kept at 60°C, and the stage of oxidation was measured by the ferric thiocyanate method (Sample, conc. 0.2%; BHT and Tocopherol, conc. 0.02%). 1, Control; 2, Tocopherol; 3, Bovine Albumin+Newlase; 4, Gluten+Newlase; 5, Egg-white Albumin+Newlase; 6, Bovine Albumin+Amano S; 7, Gluten+Amano S; 8, Egg-white Albumin+Amano S; 9, BHT.

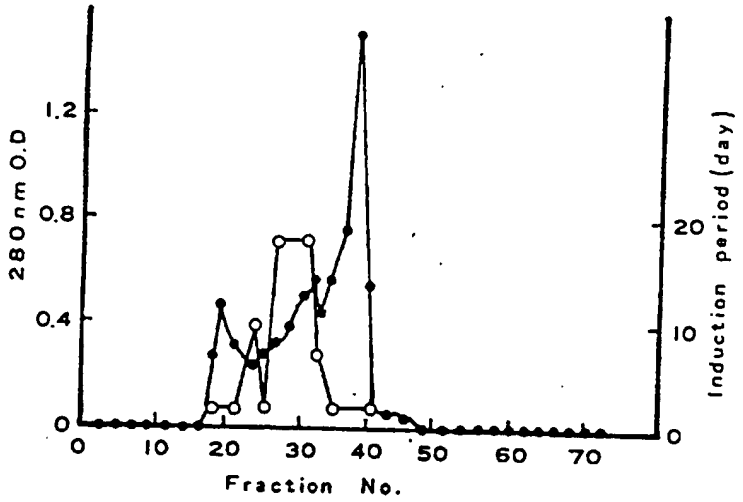

Fig. 2. Elution Profile of Egg-white Albumin Hydrolyzate by SephadexG-25 Gel Chromatography. Egg-white albumin was hydrolyzed by Amano S (B. sub. origin). Fractions of 5 ml each were collected. Induction period was defined as the days to reach absorbance 0.3 at 500 nm by the ferric thiocyanate method. ●, Absorbance at 280 nm; ○, induction period.

離を行った.

なお, 単離段階での抗酸化性テストは, 実験方法で示したスケールの 1/10 で行い, 220 nm または 280 nm の吸光度当たりの抗酸化力の比較を行った.

Sephadex G-25 を用いたときの蛋白質の溶出パターンを Fig. 2 に示した. 抗酸化活性の画分はフラクション No. 25～35 に溶出した. この画分を集め, さらにイオン交換クロマトグラフィーに供した結果を Fig. 3 に示した. イオン交換クロマト溶出物を図に示したような 6 画分に分け, モザイク荷電膜セル MC-2 MS (東ソー製) を使用して脱塩後, 抗酸化活性を調べると I-6 の画分に活性がみられた. この I-6 画分を HPLC でさらに分画

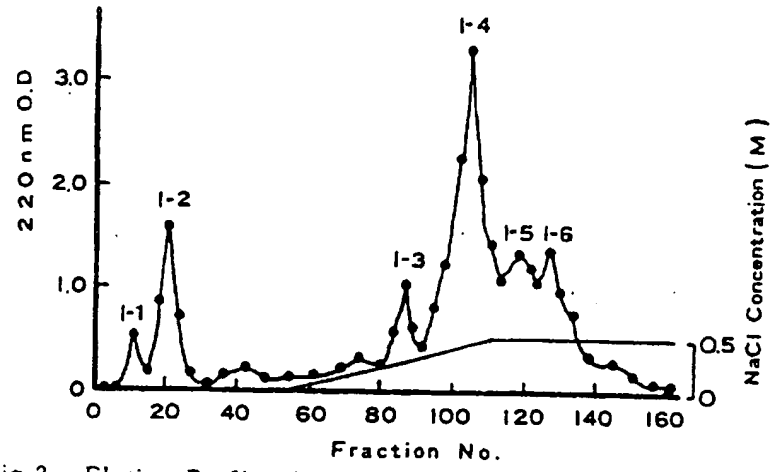

Fig. 3. Elution Profile of the Fraction No. 23～33 with Sephadex G-25 by CM Sephadex C-25 Column Chromatography. Fractions of 5 ml each were collected. ●, Absorbance at 280nm; ——, NaCl concentration.

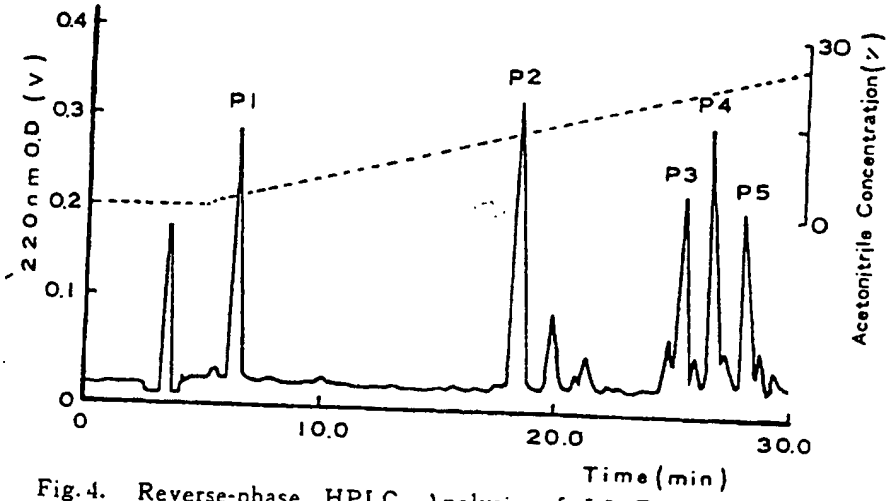

Fig. 4. Reverse-phase HPLC Analysis of I-6 Fractionated by CM Sephadex C-25 Column Chromatography. The sample was dissolved in 0.1% trifluoroacetic acid (TFA) and then put into HPLC column of TSK-gel ODS 120 T (TOSOH Corp.). Elution was done at 25℃, using a linear gradient of acetonitrile in 0.1% TFA from zero to 30% in 35 min. at a flow rate of 0.5 ml/min. ——, Absorbance at 220 nm ; ……, acetonitrile concentration.

した結果を Fig. 4 に示した. 抗酸化活性は P 1, P 2, P 3 に認められた. これらのピークを分取後, 再度 HPLC に供しても単一ピークであったことから, 単一のペプチドであると判断した. なお, 卵アルブミンの酵素分解物 5 g から, P 1 : 3.2 mg, P 2 : 1.3 mg, P 3 : 1.1 mg を単離した.

3. 単離されたペプチドのアミノ酸配列と抗酸化性について

これら P 1, P 2 および P 3 を気相シークエンサーにかけて測定し, 得られたアミノ酸配列結果を Table I に示した. また, 質量分析でも, Table I のシークエンス結果を支持するデータが得られている.

Table I. Amino Acid Sequence of Isolated Peptide

	Amino acid sequence
P 1	Ala-His-Lys
P 2	Val-His-His
P 3	Val-His-His-Ala-Asn-Glu-Asn

これら単離したペプチドを一定濃度添加したときのペプチドの抗酸化性を測定した結果を Fig. 5 に示した. 抗

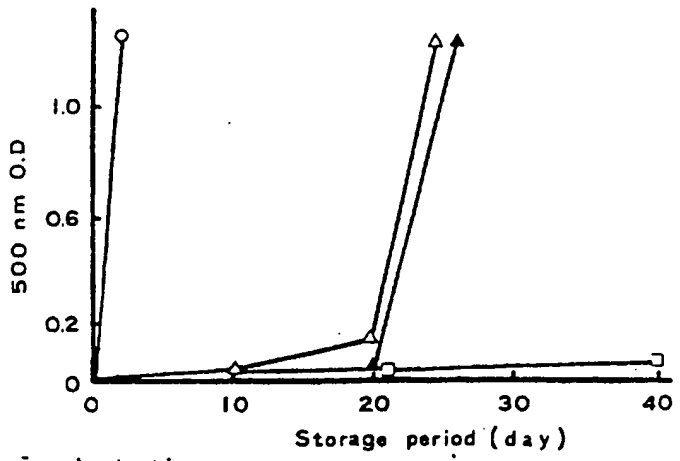

Fig. 5. Antioxidative Activity of Isolated P1, P2 and P3 Peptide. Antioxidative activity was evaluated by the ferric thiocyanate method. 9.5×10^{-5} M of each sample were added. ○, Control ; □, P1 ; △, P2; ▲, P3.

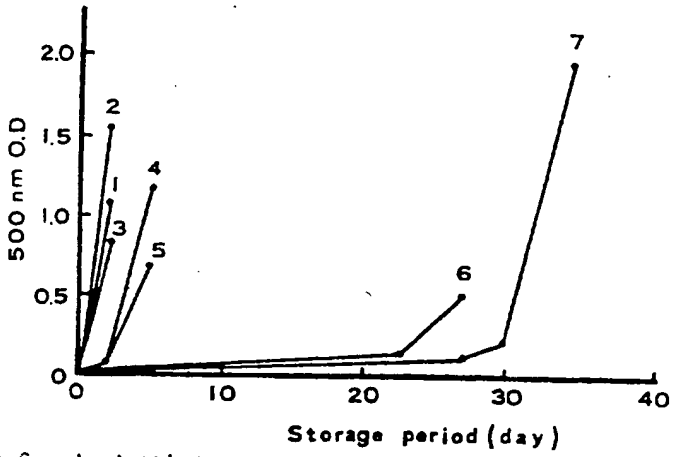

Fig. 6. Antioxidative Activity of Amino Acids, Dipeptides and Tripeptides. Antioxidative activity was evaluated by the ferric thiocyanate method. 5.2×10^{-5} M of each sample were added. 1, Control ; 2, α-tocopherol ; 3, BHT ; 4, Ala+His+Lys ; 5, His-Lys ; 6, Ala-His; 7, Ala-His-Lys.

酸化活性は，P1 が最も強く，40 日以上の誘導期間を有しており，P2，P3 はその 1/2 程度の活性であった．

以上の結果から，3つのペプチドで共通するN末端から2残基目に His を持つ構造が，抗酸化活性に関与しているものと考えられる．また，ペプチド P2，3 は抗酸化活性が同程度であることから，共通するN末端から3残基が関与し，それ以下の構造は抗酸化活性への関与が薄いと考えられる．

一方，山口ら[11]は，アラニンをN末端とするジペプチドの抗酸化性を検討し，Ala-His，Ala-Met，Ala-Tyr，Ala-Try に抗酸化性が強いことを報告している．また今回単離したペプチドで最も抗酸化性が強いものも，このN末端から Ala-His の構造を持つことから，ジペプチドとの抗酸化性の比較についても行った．Fig. 6 にその結果を示したように，Ala-His-Lys は同濃度のアミノ酸やジペプチドより強い抗酸化活性を示した．さらに His-Lys は活性が弱く，Ala-His，Ala-His-Lys に活性が強いことから，このペプチドの抗酸化性はペプチド中のアミノ酸の位置によって影響され，His がN末端から2残基目に必要なことがわかる．

また，Ala-His と Ala-His-Lys ではトリペプチドのほうが抗酸化活性が強く，3残基目の Lys も活性に関

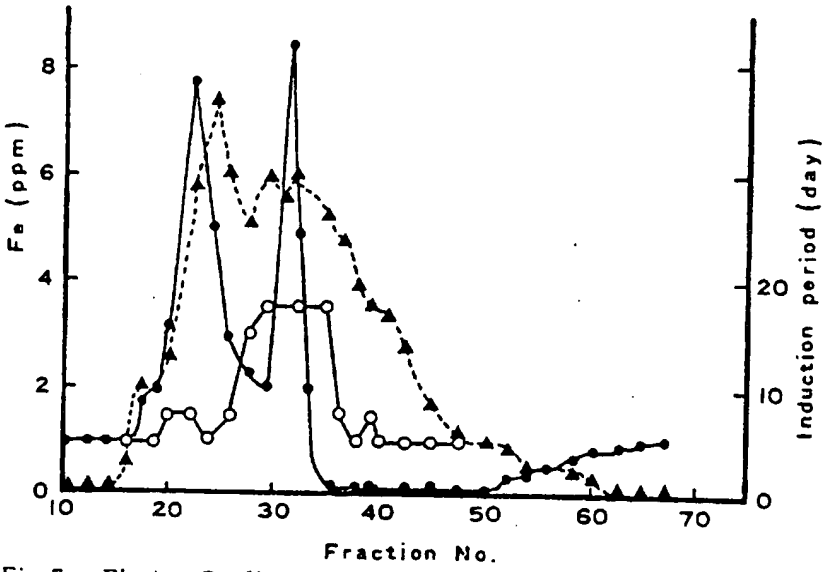

Fig. 7. Elution Profile of Egg-white Albumin Hydrolyzate with Ferrous Sulfate by Sephadex G-25 Gel Chromatography. The hydrolyzate was eluted with 70 mM acetate buffer containing 0.1 mM $FeSO_4$. Fractions of 5 ml each were collected. The iron content of the fractions was determined by inductively coupled plasma-atomic emission spectroscopy (model ICPS-50 Shimadzu Corp.). ●, Fe concentration ; ○, induction period ; ▲, absorbance at 280 nm.

与していると考えられる．この3残基目のアミノ酸の種類によって，抗酸化活性がどう変化するかは興味が持たれる点である．

4．ペプチドの抗酸化性と金属キレート能について

卵アルブミンの酵素分解物の抗酸化力と金属キレート能の関係を Fig. 7 に示した．図からわかるように，抗酸化力の強いフラクションと金属キレート能の強いフラクションは一致していた．各金属イオンに対するキレート力は，酵素分解物 1 g 当たり Fe 18 μmol, Cu 89 μmol, Zn 44 μmol であった．蛋白質の金属キレート能については，鹿本ら[16-7]が報告しているが，今回のペプチドを含む区分においても強い金属キレート能がみられた．このことから単離したペプチドの抗酸化作用が金属キレート能によることを示唆していると考えられる．

一方，Gardner[17] は，アミノ酸と過酸化脂質が反応する際に，アミノ酸の損傷によるラジカル捕捉能があることを報告している．したがって，ペプチドの抗酸化力が還元作用に起因するのか否かを確認するために，抗酸化力の強いペプチドをフェノール試薬，あるいは安定ラジカルである DPPH と反応させペプチドのラジカル捕捉能を調べた．その結果，BHT やトコフェロールでは両試薬に対して反応がみられたが，単離したペプチドではこれらの試薬との反応はまったく認められなかった．すなわち，今回単離したペプチドは，強いラジカル捕捉能を有していないと考えられる．

今まで述べてきた結果から，今回単離した，ペプチドの抗酸化作用は，金属キレート能が主体であると考えられるが，遊離のアミノ酸よりも抗酸化能が強い原因が，単に金属キレート能の増大にあるのかどうかは，今後の課題である．

要　　約

蛋白質の酵素分解物から得られたペプチドの構造と抗酸化作用について検討した．

（1）　各種蛋白質，酵素を用いて 32 種の蛋白質の酵素分解物を調製し，抗酸化性の強いものを検索した結果，蛋白質として卵アルブミン，小麦グルテン，大豆蛋白質，酵素としてアマノSを用いたときに強い抗酸化活性が認められた．

（2）　最も活性の強かった卵アルブミンの酵素分解物から，各種クロマト操作を行い抗酸化性の強いペプチド3種を単離した．

（3）　単離したペプチド3種は，Ala-His-Lys, Val-His-His, Val-His-His-Ala-Asn-Glu-Asn のアミノ酸

配列を持ち，N末端から2残基目に His を持つ構造が共通する分子量 354～819 のオリゴペプチドであった．

（4） 最も活性の強い Ala-His-Lys の抗酸化力は，同濃度の構成アミノ酸やジペプチドより強かった．

（5） 単離したペプチドの抗酸化性は金属キレート能によるものと考えられた．

本報告を終えるにあたり，ペプチドのアミノ酸配列分析をして下さいましたアプライドバイオシステムズジャパン株式会社，浅井敏夫氏に深謝いたします．

（1） 山口直彦：*New Food Industry*, 32, 68(1990).
（2） 中谷延二：日食工誌，37，569 (1990).
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